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TITLE: Genetically Modified Porcine Skin Grafts for Treatment of Severe Burn Injuries

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14. ABSTRACT

The most significant research findings in this time period include: 1) data supporting the effectiveness of skin grafts from genetically-modified swine transplanted onto full-thickness wounds on baboons when compared to the standard treatment (allogeneic skin); 2) that the use of these swine skin grafts will not sensitize the recipient to the use of a subsequent allogeneic graft if needed for prolonged wound coverage; 3) that topical immunosuppression initiated immediately after grafting may prevent graft vascularization and should therefore be delayed; 4) that systemic immunosuppression enhances the duration of survival of these swine grafts; 5) that genetically-modified porcine skin grafts prevent fluid losses as effectively as allogeneic skin grafts. These findings demonstrate that skin from genetically-modified miniature swine may provide an effective and readily-available temporary biologic cover for severe burn injuries, typical of those sustained in the battlefield.

15. SUBJECT TERMS

Burns, skin grafts, genetic modification, swine, pigskin

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INTRODUCTION:

Our study's **subject** involves "Wound infection and healing" – with special reference to "New treatment protocols, drugs, biologics, and devices to reduce wound-related infections and accelerate wound healing." In particular, we are attempting to validate the effectiveness of a novel treatment for burned warfighters using a genetically-modified pig skin graft (GalKO skin grafts). Our study's **purpose** examines whether GalKO skin grafts will perform as well as allogeneic skin grafts as a temporary biologic cover for severe burn injuries (Refs 1-4). We have utilized a non-human primate model to demonstrate the effectiveness of these grafts in a manner analogous to the clinical treatment of burn wounds in the wounded warrior. Human cadaveric allogeneic skin grafts are the current "gold standard" for temporary coverage of severe burns typical of those sustained in battle (Refs 5,6). However, our GalKO skin grafts have significant advantages over the current gold standard related to availability, cost, safety and ethical considerations (Refs 7-10). The **scope of the research** of our study will be to develop this new product for the initial treatment of severe battlefield injuries, including burns and other causes of significant skin loss, such as blast injury.

BODY:

By month 24 of this 36 month grant period, we have performed 89 grafts which comprises 66% of the entire planned work for the grant. All Specific Aims are proceeding in parallel, with Aim 1 90% complete, Aim 2 66% complete and Aim 3 33% completed.

All aims are proceeding without complications. Technical complications (mechanical avulsions) encountered in 2 skin grafts out of 89 (2%) have been corrected by modified surgical technique (sutures placed in center of graft to prevent against shear).

As such, we are *on schedule with regard to completion of the project*, and have reached 66% of our milestones by the end if Year 2 with *extremely promising outcomes.*
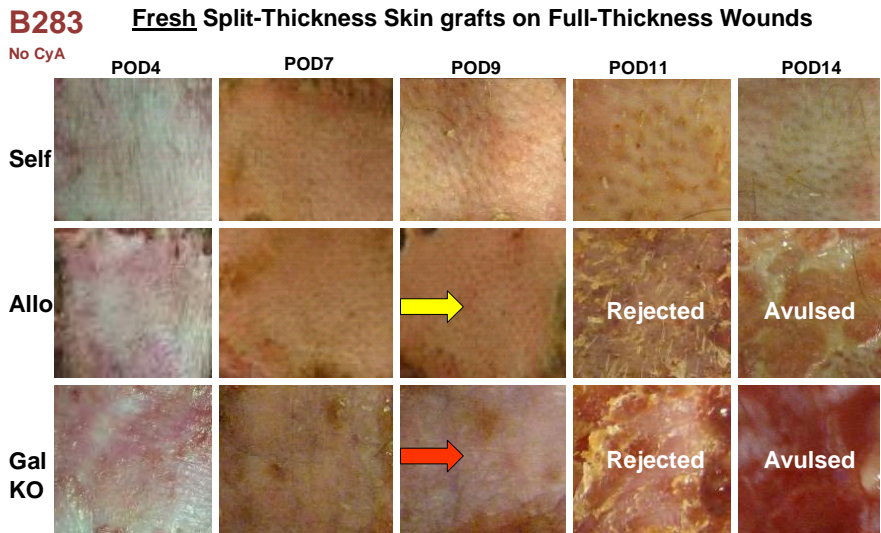
This reporting time period for Year 2 (Months 12-24) addressed the following Tasks outlined in the Approved Statement of Work (attached as Appendix 1). The tasks referenced below pertinent to Year 2 include Tasks 1c,1d,2a,2b,2c,3a,3b,3c.

Task 1c Evaluate effect of skin treatment and storage on outcome (months12-18)
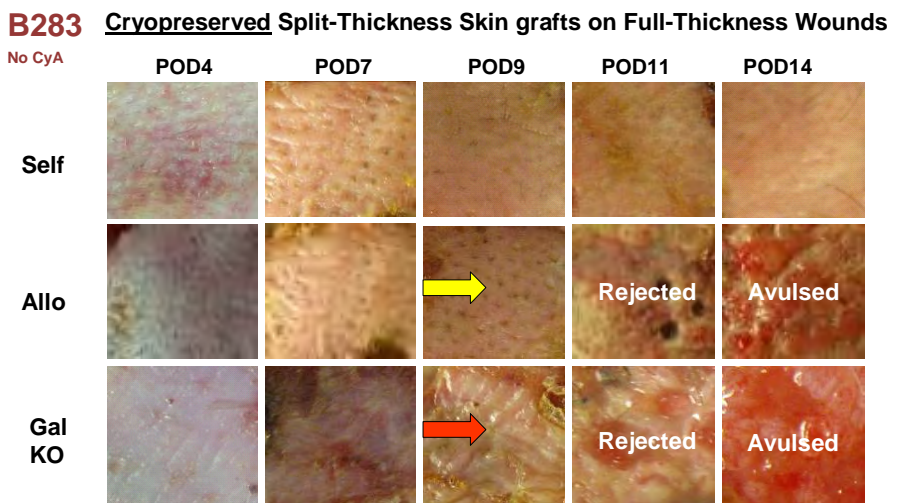
- Result: Data obtained in Year 2 demonstrates that our genetically-modified porcine skin (GalKO skin) is unaffected by cryopreservation and storage and continues to *perform as well as the current gold standard* (allograft skin (Allo)), providing up to 9 days of wound coverage. Autologous (Self) control grafts lasted indefinitely. This finding is illustrated in Figure 1 below:

Figure 1. Cryopreservation and storage of GalKO skin grafts <u>does not diminish effectiveness.</u>

1a: <u>Fresh</u> Allo (yellow arrow) and GalKO (red arrow) skin grafts provide excellent coverage up for 7-8 days.

B283
No CyA

<u>Fresh</u> Split-Thickness Skin grafts on Full-Thickness Wounds

POD4 · POD7 · POD9 · POD11 · POD14

Self / Allo / Gal KO — Rejected / Avulsed

1b: Similar coverage was obtained by Allo (yellow arrow) and GalKO (red arrow) skin grafts that were <u>cryopreserved</u>, stored for one week, thawed and grafted.


B283
No CyA

<u>Cryopreserved</u> Split-Thickness Skin grafts on Full-Thickness Wounds

POD4 · POD7 · POD9 · POD11 · POD14

Self / Allo / Gal KO — Rejected / Avulsed

Task 1d Evaluate effects of skin graft rejection on cellular and humoral responses (months 3-18)

- <u>Result:</u> Data obtained in Year 2 demonstrates that GalKO skin ***can be used successfully as a primary, first-line treatment for burns***, and will not prevent use of additional standard graft materials (allografts) from being used later, if longer temporary coverage is needed. Our GalKO grafts will not sensitize the recipient and prevent successful use of subsequent allogeneic grafts. ***This finding is clinically very important, as often burn centers require a few days to a week to acquire cadaver allograft-*** in the meantime, GalKO skin could be used until standard cadaver allografts arrived. This finding is illustrated in Figure 2 below:

Figure 2. Above: A. First-line treatment of GalKO skin shown here- well-vascularized, providing coverage on Day 7. **B.** Allogeneic skin shown on Day 17 on the same baboon. This graft was placed on Day 10 after rejection and avulsion of the first GalKO graft. The graft is shown here providing coverage for an additional 7 days (shown here at Day 17).

Allogeneic skin rejects at a normal rate following Xenogeneic skin grafting

A. GalKO   Day 7

B. Allo   Day 17 (Allo graft placed on Day 10 after previous GalKO graft had rejected)
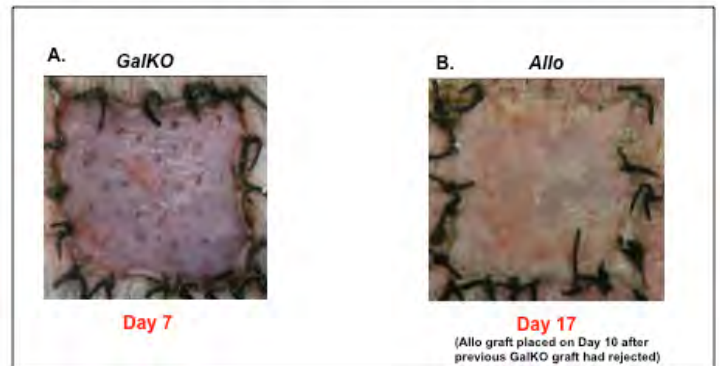
Task 2a 28-day course of calcineurin inhibitor to group 1 (months 18-24)

- Result: The length of coverage of both genetically-modified swine skin and standard allogeneic skin are prolonged by approximately 2 days by systemic calcineurin inhibitor administration. See Figure 3 below.

Task 2b Topical calcineurin inhibitor to group 2 (months 18-30)

- Result: Topical immunosuppressive cream, administered immediately after surgery, prevents early vascularization and results in early graft loss (average graft survival: 2 days). See Figure 3 below.

Task 2c Evaluate immune effects of skin graft on cellular and humoral responses (months 18-30)

- Result: Topical immunosuppression cream, administered on post-graft days 4-14, allows vascularization and results in ***prolongation*** of graft survival (to an average of 11 days). See Figure 3 below:

Figure 3. Comparison of survival of GalKO and Allo grafts with various methods of immunosuppression (None vs. Intravenous vs. Topical (applied days 0-4) vs. Topical (applied day 4-10)
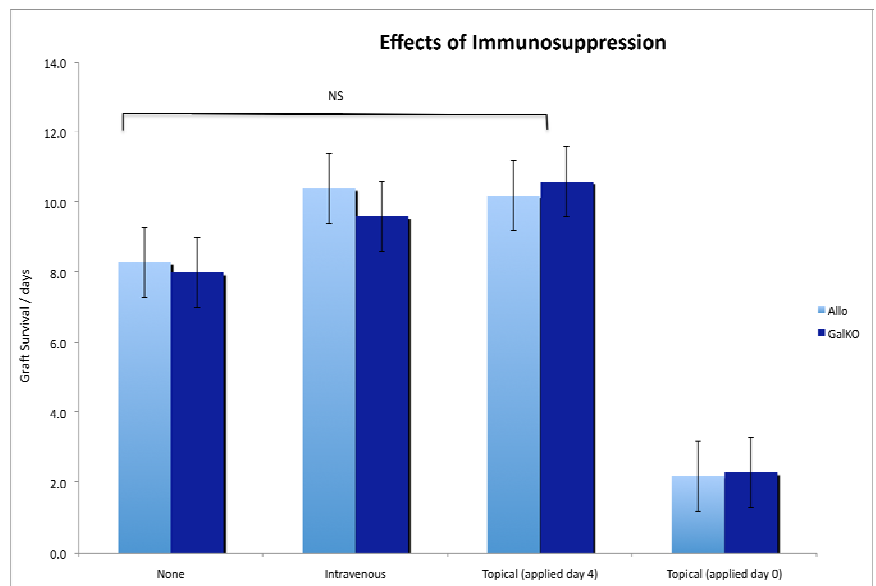
Task 3. Compare acute and long term results of xeno skin grafts with autologous and allogeneic skin grafts

Task 3a Examine wound discharge to determine fluid and electrolyte loss (months 12-36)

- Result: Preliminary experiments indicate that GalKO skin grafts protect against fluid and loss as effectively as allografts. Electrolyte losses were also equally prevented by Self, Allo and GalKO grafts (data not shown). See Figure 4 below:
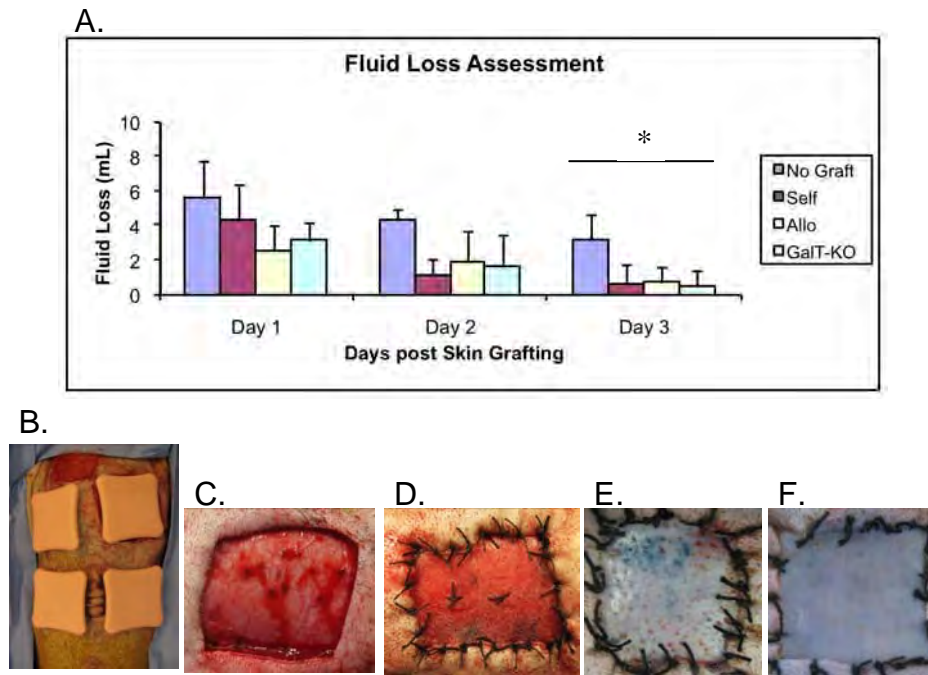

Figure 4. **A.** Following full-thickness wounds, abundant fluid loss occurs if no coverage is provided with skin grafting. Fluid loss remains high on day 3. In contrast, following skin grafting with Self, Allo or GalKO skin, fluid loss rapidly decreases until day 3. By day 3, grafted wounds lose significantly less fluid and electrolytes. No significant differences are seen in fluid loss following Allo and GalKO coverage. **B.** Design of non-human primate wound fluid collection in vivo assay. **C.** Full-thickness wound without coverage. **D.** Full-thickness wound with Self graft. **E.** Full-thickness wound with Allo graft. **F.** Full-thickness wound with GalKO graft.

Task 3b Determine effect of wound coverage incidence and severity of infection (months 12-36)

- Result: Preliminary experiments indicate that GalKO skin grafts protect against wound infection as effectively as allografts. See Figure 5 below:

Figure 5. The observed rate of wound infection in preliminary experiments was 2%. There was no significant difference between wounds covered with allografts and GalKO grafts.
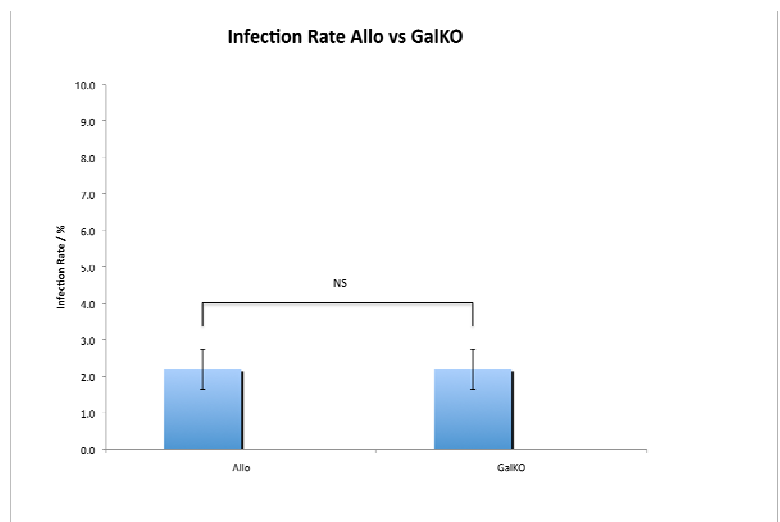
Task 3c Examine regrowth of self skin in the wound bed to determine cosmetic outcome (months 12-36)

- Result: Preliminary experiments indicate that wounds covered temporarily with GalKO skin grafts have a comparable cosmetic outcome to wounds covered temporarily with allogeneic skin. See Figure 6 below:

Figure 6. Representative slide of comparable cosmetic outcome of wounds treated with temporary grafts of GalKO or Allo. *Left:* Permanent, autologous grafts (Self- positive control); *Center:* GalKO temporary graft wound result; *Right:* Allo temporary graft wound result.
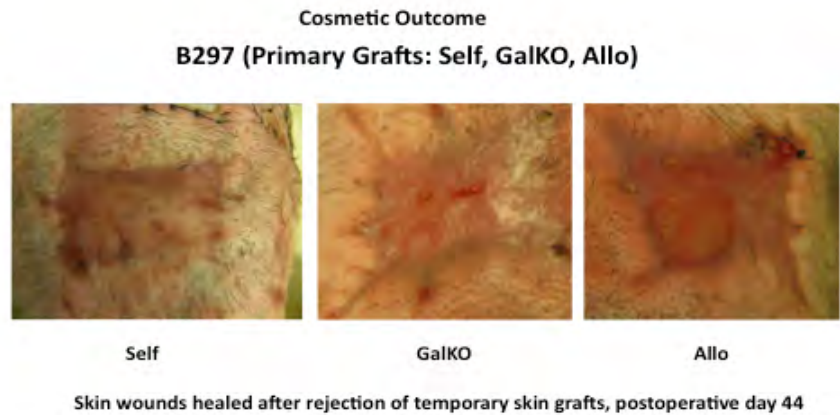
KEY RESEARCH ACCOMPLISHMENTS (YEAR 2):

- Cryopreservation and storage of genetically-modified (GalKO) porcine skin grafts does not diminish their effectiveness.

- GalKO skin grafts can be used as a primary, first-line treatment without "burning any bridges"-(i.e. additional standard allogeneic grafts can be used later if longer wound coverage is needed)

- Intravenous immunosuppression moderately enhances the duration of graft coverage

- Topical immunosuppression moderately enhances the duration of graft coverage, but should be delayed until vascularization occurs (postgraft day 4)

- GalKO skin grafts prevent fluid and electrolyte losses and wound infections as effectively as allogeneic skin grafts

- Cosmetic outcomes of wounds covered with GalKO skin grafts are comparable to those treated with allogeneic grafts.

REPORTABLE OUTCOMES:

Reportable outcomes that have resulted from this research are listed below. See also Appendices 2-4.

- Manscripts/Presentations/Abstracts

1. Leto Barone AA, Torabi R, Hanekamp JS, Yamada K, Sachs DH, Cetrulo CL Jr. Genetically-modified porcine skin treatment of severe burns. *Burns,* Manuscript in preparation, June 2011.

2. Leto Barone, AA, Torabi R, Albritton AB, Mallard, C, Leonard DA, Kurtz JM, Sachs DH, Cetrulo CL Jr. GalT-knockout porcine xenoskin grafts are equivalent to allogeneic skin grafts in nonhuman primates. *Plastic & Reconstructive Surgery*, Manuscript in preparation, June 2011

3. Albritton AB, Leto Barone AA, Torabi R, Mallard C, Leonard DA, Kurtz JM, Sachs DH, Cetrulo CL Jr. Absence of cross-sensitization between GalT-KO skin and allogeneic skin may allow prolonged temporary coverage of severely burned patients. *Science Translational Medicine,* Manuscript in preparation, June 2011

4. Mallard C, Leto Barone AA, Torabi R, Albritton AB, Leonard DA, Kurtz JM, Sachs DH, Cetrulo CL Jr. Recent advances in the temporary coverage of severe burns. *Journal of Burn Care and Research*, Manuscript in preparation, June 2011

5. Leto Barone, A.A., Torabi R., Weiner J, Albritton A.B., Mallard, C., Tang Y., Hanekamp J.S., Smith R.N., Yamada K., Kurtz J., Sachs D.H., Cetrulo C.L. Jr., Austen W.G. *Absence of Cross-Sensitization Between GalT-KO Skin and Allogeneic Skin May Allow Prolonged Temporary Coverage of Severely Burned Patients.* Presented at: New England Society of Plastic Surgeons, Brewster, MA, June 2011.

6. Leto Barone A.A., Sachs D.H., Weiner J., Torabi R., Hanekamp J.S., Yamada K., Cetrulo C.L. *Comparison of Survival of GalT-KO Pig Skin to Allogeneic Skin on Baboons: the Role of Graft Bed Depth and of Cyclosporine Treatment.* Presented at: XXIII International Congress of the Transplantation Society; British Columbia, Canada, August 2010.

7. Leto Barone A.A., Torabi R., Hanekamp J.S.,Weiner J., Yamada K., Sachs D.H., Cetrulo C.L. *Genetically Modified Porcine Skin Treatment of Severe Burns: Non-Human Primate Data.* Presented at: 27th Army Science Conference; Orlando, FL, December 2010.

8. Leto Barone A.A., Weiner J, Hanekamp JS, Yamada K, Cetrulo CL, Sachs DH. *Comparable Graft Survival of GalT-KO Pig Skin and Allogeneic Skin on Baboons.* Presented at: 55th Plastic Surgery Research Council; San Francisco, CA, May 2010.

9. Leto Barone A.A., Sachs D.H., Weiner J., Torabi R., Hanekamp J.S., Yamada K., Cetrulo C.L.. (2010, August). *Comparison of Survival of GalT-KO Pig Skin to Allogeneic Skin on Baboons: the Role of Graft Bed Depth and of Cyclosporine Treatment.* Abstract: XXIII International Congress of the Transplantation Society; British Columbia, Canada.

10. Leto Barone A.A., Torabi R., Weiner J., Albritton A., Mallard C., Tang Y., Hanekamp J.S., Smith R.N., Yamada K., Kurtz J.M., Sachs D.H., Cetrulo C.L. Jr. (2011, June) *Absence of Cross-Sensitization Between GalT-KO Skin and Allogeneic Skin May Allow Prolonged Temporary*

Coverage of Severely Burned Patients. Abstract: 52nd Annual Meeting of the New England Society of Plastic and Reconstructive Surgeons

- Patents and licenses applied for and/or issued – Covered by our GalT-KO patents

- Degrees obtained that are supported by this award- None

- Development of cell lines- None

- Tissue or serum repositories- None

- Infomatics such as databases and animal models, etc.- None

- Funding applied for based on work supported by this award-

  Radbeh Torabi, M.D.- Massachusetts General Hospital/Harvard Medical School NIH T32 Research Training Grant Scholarship

  Angelo Leto Barone, M.D.- Harvard Medical School Tosteson Fellowship, 2010-2011.

- Employment or research opportunities applied for and/or received based on experience/training supported by this award-

  o Josef Kurtz, Ph.D.- Instructor in Immunology, Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School
  o Angelo Leto Barone, M.D.- Research Fellow, Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School
  o Radbeh Torabi, M.D.- Research Fellow, Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School
  o Christopher Mallard, B.S.- Research Technician, Transplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School

CONCLUSION:

Summary of Results

The **importance and implications of the completed research** in Year 2 include:

1) Demonstration that cryopreservation, storage and thaw of GalKO grafts does not affect their performance: the <u>implication</u> here is that these *grafts can be frozen and stored in forward areas*, readily-available to be used immediately upon receiving burn casualties.

2) The use of these swine skin grafts *will not sensitize the recipient to the use of a subsequent allogeneic graft prolonged wound coverage is needed*: the <u>implication</u> here is that these grafts will lengthen the time of temporary coverage possible, providing a significant window of opportunity for a severely burned warrior to recover and perhaps survive an otherwise unsurvivable burn.

3) When topical immunosuppression is applied immediately after grafting (day 0), it prevents graft vascularization and leads to graft avulsion.

4) When topical immunosuppression is applied on day 4 after vascularization of grafts occurs, topical immunosuppression **prolongs** graft survival.

5) Systemic immunosuppression enhances the duration of survival of these swine grafts; the <u>implication</u> here is that systemic immunosuppression may be used to prolong coverage until standard grafts are available for further coverage as needed.

6) GalKO skin grafts prevent fluid losses as effectively as allogeneic skin grafts.

7) GalKO skin grafts prevent infection as effectively as allogeneic skin grafts.

8) GalKO skin grafts have comparable cosmetic outcomes to allogeneic skin grafts.

These findings demonstrate that skin from genetically-modified miniature swine may provide an effective and readily-available temporary biologic cover for severe burn injuries, typical of those sustained in the battlefield.

"So What" Section:

Evaluation of the results obtained during the first 18 months of this project yielded strong evidence supporting the clinical applicability of GalKO porcine skin grafts. We are closer to providing a critical tool for the care of blast-injured or burned soldiers: a frozen, readily-available military burn dressing that can be used as a lifesaving temporary skin graft for immediate, sterile coverage of critically-injured areas of a blast-wounded or flame-burned soldier's body. Immediate coverage of such severe wounds would prevent life-threatening infection and fluid/electrolyte loss while the combatant is evacuated to tertiary-care centers for definitive treatment (Refs1-5).

The GalKO porcine skin graft would provide wound coverage for as many as 7-10 days post-injury before requiring either extended temporary coverage with a cadaver allograft or definitive replacement with a permanent autograft.

This technology would replace or provide an adjunct to the current method of utilizing human cadaver allograft skin as a temporary dressing- an extremely effective technique that is underutilized due to a lack of availability, portability, cost-effectiveness, as well as ethical and infectious disease concerns associated with the use of human tissue.

Furthermore, our recent data suggests a novel paradigm that could be employed to lengthen the time of temporary coverage in a critically-ill soldier: currently, a cadaver allograft will last only 7-10 days, before autografting is necessary, and in these 7-10 days a critically-ill soldier may not yet be stable enough for autografting- this clinical scenario is common in severe burns. A second round of cadaver allografting is not possible due to presensitization, which would result in hyperacute rejection of any subsequent cadaver allograft skin. However, we have demonstrated that GalT-KO xenogeneic skin will not presensitize to allogeneic skin- therefore, GalT-KO xenogeneic skin could be used for the first 7-10 days followed by a cadaver allograft for the subsequent 7-10 days while intensive care measures are taken to stabilize the soldier for autografting. **This approach would double the window for stabilization of criticall-ill patients.**

We intend to breed and maintain a herd of GalT-KO donor swine for this purpose as a new approach to the initial treatment of severe battlefield injuries, and are currently in the commercial development phase with a biotechnology company specializing in bringing xenotransplantation products to market (AXI, Inc.). Our goal will be to demonstrate pre-clinical proof-of-concept and increase our current Technology Readiness Level from 5 to 6 by the end of this 3-year grant.

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10. Leon-Villapalos J, Eldardiri M, Dziewulski P. The use of human deceased donor skin allograft in burn care. Cell Tissue Bank. 2010 Feb;11(1):99-104.

APPENDIX 1

Approved Statement of Work

Approved Statement of Work

Milestone #1 Develop animal protocol and submit for IACUC and ACURO approval (Month 2)

Specific Aim 1: Compare the survival of skin grafts to baboons from GalT-KO swine to the survival of skin grafts from unmodified swine or from allogeneic baboons and study the response of these grafts by gross examination, by histology and by evaluation of the cellular and humoral immune responses evoked. (Month 3 - Month 18)

Task 1. Develop and compare survival of GalT-KO swine skin, normal swine skin and allogeneic skin grafts to baboons from GalT-KO swine (Month 3-Month 18)

1a. Evaluate effect of immunosuppression on outcome (month 3-12)

1b. Evaluate role of graft technique (split vs. full thickness) on outcome (month 3-12)

1c. Evaluate effect of skin treatment and storage on outcome (month 12-18)

1d. Evaluate effects of skin graft rejection on cellular and humoral responses (month 3-18)

Milestone #2 Optimize GalT-KO Swine skin graft survival on baboons (Month 18)

Specific Aim 2: Evaluate the effectiveness of treatments of the skin graft and of the recipient designed to prolong graft survival, including topical and systemic administration of immunosuppressive agents. (Month 18 – Month 30)

Task 2. Systematic administration of immunosuppressive agents

2a. 28-day course of calcineurin inhibitor to group 1 (month 18-24)

2b. Topical calcineurin inhibitor to group 2 (month 18-30)

2c. Evaluate immune effects of skin graft on cellular and humoral responses (month 18-30)

Milestone #3 Determine effect of systemic immunosuppression with Tacrolimus: (month 24)

Milestone #4 Determine effect of topical immunosuppression (Month 30)

Specific Aim 3. Determine the consequences of wound coverage by GalT-KO skin grafts in comparison to the coverage with autologous (or syngeneic) skin grafts, in terms both of physiologic parameters and of the cosmetic outcome following subsequent autografts. (Month 12-Month 36)

Task 3. Compare acute and long term results of xeno skin grafts with autologous and allogeneic skin grafts

3a. Examine wound discharge to determine fluid and electrolyte loss (month 12-36)

3b. Determine effect of wound coverage incidence and severity of infection (month 12-36)

3c. Examine regrowth of self skin in the wound bed to determine cosmetic outcome (month 12-36)

Milestone #5 Comparison of fluid and electrolyte loss with each tested skin coverage (Month 30)

Milestone #6 Comparison of infection rates following each tested skin coverage (Month 36)

Milestone #7 Determine cosmetic outcome following each tested skin coverage: (Month 36)

Milestone #8 Publications (Month 36)

APPENDIX 2

Poster Presentation at 27th Army Science Conference,
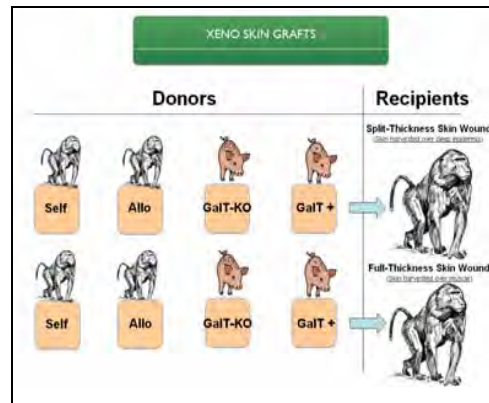Orlando, Florida, November, 2010

Genetically-modified GalT-KO Porcine Skin as a First-line Treatment in Severely-Burned Patients in the War Zone
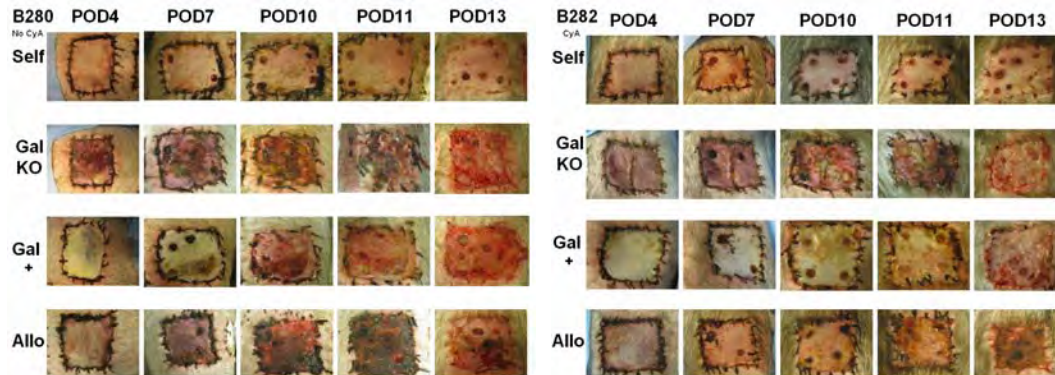
Curtis L Cetrulo, Jr, MD[1, 2], Angelo A Leto Barone, MD[1, 2], Joshua Weiner[1], Radbeh Torabi, John S Hanekamp, MD, PhD[1], Kazuhiko Yamada, MD, PhD[1], David H Sachs, MD[1]

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[2]Division of Plastic and Reconstructive Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA

INTRODUCTION: Approximately 5-10% of all combat casualties sustain severe burns, and 6% of these injuries are fatal. Immediate eschar excision and skin grafting has been clearly demonstrated to decrease mortality. Excision and grafting is utilized for full-thickness burns in the immediate period after injury to prevent infection and severe metabolic impairment secondary to fluid and electrolyte loss. The ideal material for grafting is autologous skin, taken from a non-burned region of the patient's own skin. However, by definition, the supply of unburned autologous skin in large total body surface area burns is limited. Allogeneic cadaver grafts are the current gold standard for temporary burn coverage, but this modality is limited by the infrequent availability of an adequate supply of graft material. A readily-available alternative material for temporary coverage would provide a significant resource for the care of severely burned soldiers. We have developed a genetically-modified miniature swine herd that may provide such material- the α-1,3-galactosyltransferase knockout (GalT-KO) swine. In contrast to wild-type swine, these animals do not express the cell-surface antigen (the Gal antigen) associated with hyperacute rejection across xenogenic (pig-to-primate) barriers. We hypothesized that GalT-KO porcine skin may be effective for the temporary coverage of severe burns in primates.

XENO SKIN GRAFTS — Donors: Self, Allo, GalT-KO, GalT +. Recipients: Split-Thickness Skin Wound, Full-Thickness Skin Wound.

METHODS: Eight naïve baboons were transplanted with split-thickness skin grafts from allogeneic and xenogeneic porcine donors. Group 1 (n=2) received simultaneous skin grafts over split-thickness wounds from self (control), allogeneic baboon, GalT-KO swine, and Gal+ swine. Group 2 (n=4) underwent the same transplantation over full-thickness skin wounds. One baboon in group 1 and two baboons in group 2 also received Cyclosporine A (CyA; 13-15 mg/kg/day I.M. for target blood levels of 200-400 ng/mL). Graft viability was assessed by clinical observation and by pathologic evaluation of punch-biopsies taken on POD 4, 7, 11 and 14. Two baboons from group 2 underwent both fresh and freeze-thawed split-thickness skin grafts over full thickness wounds to compare engraftment, viability and overall outcome. Finally, the two baboons in group 1 were retransplanted following rejection of the primary grafts to investigate whether sensitization to the various graft types had occurred. These animals were retransplanted with self, allo, fresh Gal-KO skin and frozen-thawed GalT-KO skin. In vitro assays (Mixed Lymphocytic Reaction, ELISAs, and flow cytometry) were performed prior to and at multiple time points following transplantation.

B280 No CyA — Self, Gal KO, Gal +, Allo at POD4, POD7, POD10, POD11, POD13.

B282 CyA — Self, Gal KO, Gal +, Allo at POD4, POD7, POD10, POD11, POD13.

Experimental Observations

1st graft: GalT + → Preformed Ab Response → White Graft. GalT-KO → No preformed Ab → Sensitization. Allo → No preformed Ab → Sensitization. 2nd graft → Post regrafting.

RESULTS: Baboons in all groups showed comparable survival of GalT-KO and allogeneic baboon skin grafts. In group 1 (split-thickness wound beds), both GalT-KO grafts and allogeneic grafts remained viable until POD 7, and in group 2 (full-thickness wound beds), both GalT-KO grafts and allogeneic grafts remained viable until POD 11. With CyA treatment, GalT-KO and allograft skin survival were prolonged in both groups. In all animals, Gal+ xenografts were hyperacutely rejected (white grafts) by POD 1. Self grafts survived indefinitely in all cases. No major differences were noted between freshly harvested and freeze-thawed xenografts. GalT-KO and allogeneic retransplants were rejected by POD4. Sensitization was supported by antibody FACS, showing increase of the anti-nonGal Ig and anti-allo Ig.

Split -Thickness Skin Grafts over full thickness wounds. Gal KO Fresh, Gal KO Frozen.

CONCLUSIONS: 1) GalT-KO skin xenografts do not exhibit hyperacute rejection typical of wild-type (Gal+) porcine skin grafts when grafted onto either split or full thickness graft beds. 2) GalT-KO skin xenografts exhibit comparable survival to allografts, surviving up to 11 days before rejection; 3) Treatment with CyA prolonged the survival of both GalT-KO and allogeneic skin grafts. 4) No major differences in survival between fresh and frozen GalT-KO skin was observed. 5) In sensitized animals, regrafting with both GalT-KO skin and allogeneic grafts resulted in accelerated failure of the grafts presumably due to sensitization.

Our data suggests that GalT-KO skin, either freshly harvested or freeze-thawed, may provide an effective treatment for temporary coverage of both split- and full-thickness skin defects analogous to severe burn injuries. The sensitization process following the first xenotransplant renders a subsequent retransplant with GalT-KO skin unsuitable for coverage. Further experiments will be oriented to investigate whether allogeneic skin could be used following first-line GalT-KO skin xenotransplantation in sensitized patients. This approach may provide prolonged temporary coverage and metabolic stabilization of burned warfighters.

APPENDIX 3

Poster Presentation at Massachusetts General Hospital-Harvard
Medical School Science Conference, Boston, Massachusetts
January 2011

Primary xenogeneic porcine α-1,3-galactosyltransferase knockout skin grafts do not elicit a strong anti-allo humoral response in non-human primate model

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Background

The current protocol for temporary local skin coverage of deep burns is skin grafting from cadaveric allogeneic donors. However allogeneic skin grafts are limited in availability. Our group has previously shown that use of skin from alpha-1,3-galactosyltransferase knockout (GalKO) swine, which are unable to produce the α-galactosyl epitope, avoids the classic hyperacute rejection observed in porcine to primate xenotransplantation, leading to prolonged graft survival similar to allogeneic grafts. We have shown that primary allogeneic and GalKO porcine skin grafts placed onto baboons are rejected in a similar time frame of 7-11days.

Current Work / Experimental Design

The aim of this study is to investigate if the grafting of a GalKO xenograft precludes the subsequent grafting of an allogeneic skin graft. A baboon was given primary split thickness skin grafts of self and GalKO skin over full thickness defects. Following primary GalKO skin rejection, secondary grafts of GalKO and allogeneic skin where placed on different areas of the baboon and assessed for time course of rejection. Grafts were assessed daily for rejection, and production of anti-xeno and anti-allo IgM and IgG antibodies were measured from the serum by flow cytometry at various times post-transplant.

Conclusions

In a non-human primate model, primary xenogeneic GalKO skin grafts are followed by a high anti-xeno (non Gal) humoral response, without strong evidence for cross-sensitization to allogeneic antigens as measured by flow cytometry.

These data suggest that xenogeneic skin grafts may provide an early first-line clinical approach for large total body surface area burns that does not preclude the use of allografts in subsequent treatments when secondary grafting is required.

Figure 1: Rejection of primary xenogeneic skin graft (GalKO) does not lead to accelerated rejection of a secondary allogeneic skin graft.
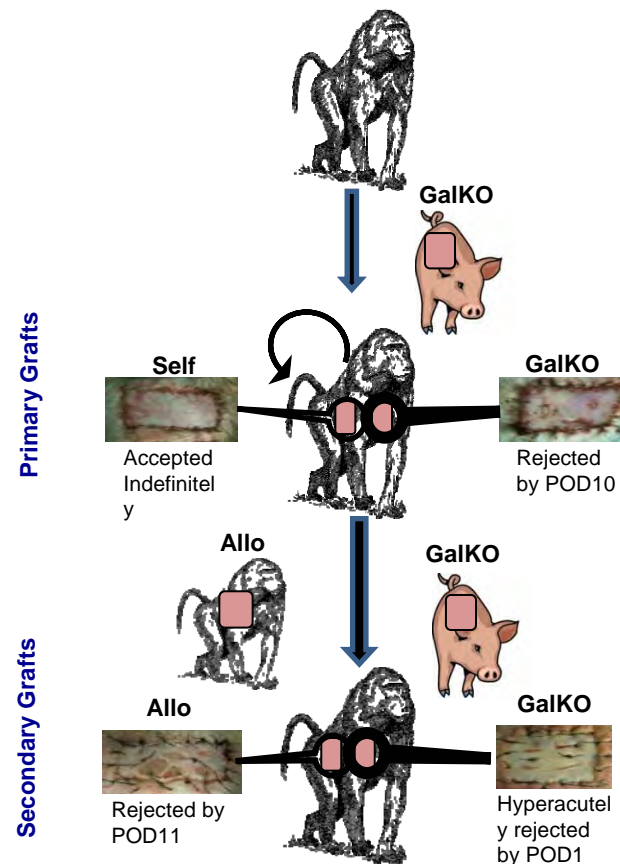

Figure 1: GalKO skin grafts were rejected by day 10 post placement, while control self skin was accepted indefinitely. A second GalKO graft placed 41 days after primary grafting was rejected within one day, demonstrating a sensitized response. Allogeneic skin placed at the same time was rejected by day 11, similar to previous results observed in naïve animals. This data suggests that the rejection of a primary xenogeneic skin graft does not presensitize the recipient to a secondary allogeneic graft.

Figure 2: Flow cytometric analysis of anti-xeno and anti-allogeneic IgM and IgG antibodies following rejection of skin grafts.
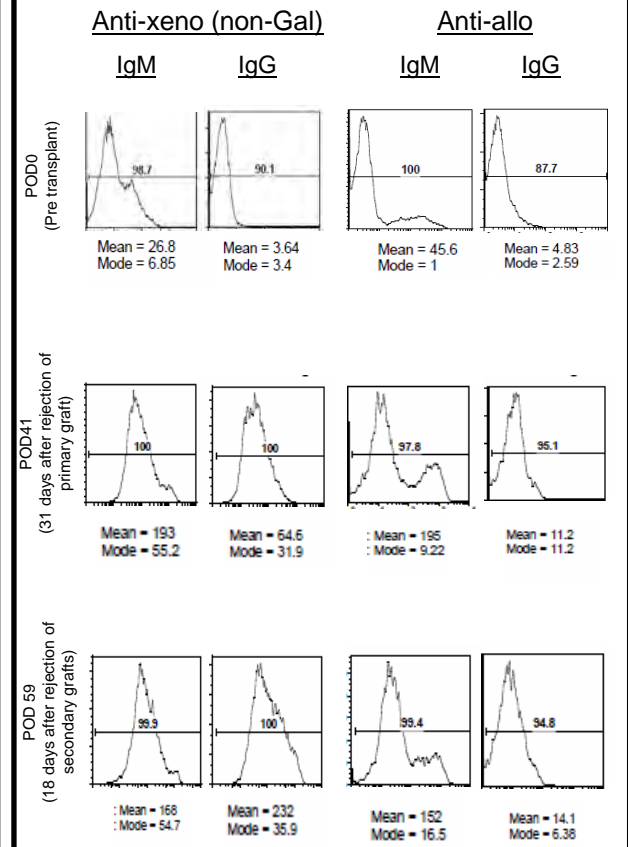

Figure 2: Transplant recipients where assessed to see if GalKO xenogeneic porcine skin transplantation elicits a strong anti-allo antibody response. Serum was collected prior to transplant (POD 0; top), POD 41 (31 days after rejection of primary GalKO graft; middle), and POD 59 (18 days after rejection of allogeneic graft; bottom). Following rejection of primary GalKO skin, high levels of anti-xeno IgM and IgG antibody were detected, but no significant anti-allo antibody was observed. Following rejection of secondary grafts, high levels of anti-xeno antibody were still detected but no anti-allo antibody was observed.

APPENDIX 4

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http://www.armyscienceconference.com/sessions/sessionk.htm

GENETICALLY-MODIFIED PORCINE SKIN AS A FIRST-LINE TREATMENT FOR SEVERELY BURNED PATIENTS IN THE WAR ZONE

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1. ABSTRACT

INTRODUCTION: Approximately 5-10% of all combat casualties sustain severe burns, and 6% of these injuries are fatal. Immediate eschar excision and skin grafting has been clearly demonstrated to decrease mortality. Excision and grafting is utilized for full-thickness burns in the immediate period after injury to prevent infection and severe metabolic impairment secondary to fluid and electrolyte loss. The ideal material for grafting is autologous skin, taken from a non-burned region of the patient's own skin. However, by definition, the supply of unburned autologous skin in large total body surface area burns is limited. Allogeneic cadaver grafts are the current gold standard for temporary burn coverage, but this modality is limited by the infrequent availability of an adequate supply of graft material. A readily-available alternative material for temporary coverage would provide a significant resource for the care of severely burned soldiers. We have developed a genetically-modified miniature swine herd that may provide such material- the α-1,3-galactosyltransferase knockout (GalT-KO) swine. In contrast to wild-type swine, these animals do not express the cell-surface antigen (the Gal antigen) associated with hyperacute rejection across xenogenic (pig-to-primate) barriers. We hypothesized that GalT-KO porcine skin may be effective for the temporary coverage of severe burns in primates.

METHODS: Eight naïve baboons were transplanted with split-thickness skin grafts from allogeneic and xenogeneic porcine donors. Group 1 (n=2) received simultaneous skin grafts over split-thickness wounds from self (control), allogeneic baboon, GalT-KO swine, and Gal+ swine. Group 2 (n=4) underwent the same transplantation over full-thickness skin wounds. One baboon in group 1 and two baboons in group 2 also received Cyclosporine A (CyA; 13-15 mg/kg/day I.M. for target blood levels of 200-400 ng/mL). Graft viability was assessed by clinical observation and by pathologic evaluation of punch-biopsies taken on POD 4, 7, 11 and 14. Two baboons from group 2 underwent both fresh and freeze-thawed split-thickness skin grafts over full thickness wounds to compare engraftment, viability and overall outcome. Finally, the two baboons in group 1 were retransplanted following rejection of the primary grafts to investigate whether sensitization to the various graft types had occurred. These animals were retransplanted with self, allo, fresh Gal-KO skin and frozen-thawed GalT-KO skin.

In vitro assays (Mixed Lymphocytic Reaction, ELISAs, and flow cytometry) were performed prior to and at multiple time points following transplantation.

RESULTS: Baboons in all groups showed comparable survival of GalT-KO and allogeneic baboon skin grafts. In group 1 (split-thickness wound beds), both GalT-KO grafts and allogeneic grafts remained viable until POD 7, and in group 2 (full-thickness wound beds), both GalT-KO grafts and allogeneic grafts remained viable until POD 11. With CyA treatment, GalT-KO and allograft skin survival were prolonged in both groups. In all animals, Gal+ xenografts were hyperacutely rejected (white grafts) by POD 1. Self grafts survived indefinitely in all cases. No major differences were noted between freshly harvested and freeze-thawed xenografts. GalT-KO and allogeneic retransplants were rejected by POD4. Sensitization was supported by antibody FACS, showing increase of the anti-nonGal Ig and anti-allo Ig.

CONCLUSIONS: 1) GalT-KO skin xenografts do not exhibit hyperacute rejection typical of wild-type (Gal+) porcine skin grafts when grafted onto either split or full thickness graft beds. 2) GalT-KO skin xenografts exhibit comparable survival to allografts, surviving up to 11 days before rejection; 3) Treatment with CyA prolonged the survival of both GalT-KO and allogeneic skin grafts. 4) No major differences in survival between fresh and frozen GalT-KO skin was observed. 5) In sensitized animals, regrafting with both GalT-KO skin and allogeneic grafts resulted in accelerated failure of the grafts presumably due to sensitization.

Our data suggests that GalT-KO skin, either freshly harvested or freeze-thawed, may provide an effective treatment for temporary coverage of both split- and full-thickness skin defects analogous to severe burn injuries. The sensitization process following the first xenotransplant renders a subsequent retransplant with GalT-KO skin unsuitable for coverage. Further experiments will be oriented to investigate whether allogeneic skin could be used following first-line GalT-KO skin xenotransplantation in sensitized patients. This approach may provide prolonged temporary coverage and metab1olic stabilization of burned warfighters.

2. INTRODUCTION

In the Iraq and Afghanistan theaters of war, 5-10% of combat casualties sustain severe burns of total body

surface area (TBSA) >50%, and 6% of these injuries are fatal[1,2]. Treatment of such burns has evolved in recent decades. Early excision of burned skin with replacement by autologous grafted skin has decreased mortality by preserving the skin's barrier function and preventing severe fluid loss and hypovolemia, as well as electrolyte, temperature and pH imbalances that predispose to infection and multisystem organ failure. However, the supply of unburned autologous skin is limited in such patients, driving the search for alternative means of temporary covering to preserve barrier function[3-10].

Cultured autologous keratinocytes and various artificial coverings have been described to this end; however, both approaches have disadvantages[11-13]. Cultured autologous keratinocytes are expensive and require weeks to grow before application, and artificial coverings such as Integra, are expensive and prone to infection. Currently, allografted skin harvested from a cadaver donor and stored frozen in tissue banks represents the gold standard for temporary skin grafts. These grafts undergo inosculation (the formation of reconnections between host vessels in the burn wound bed and vessels in the allograft skin itself) within 2-3 days and are therefore resistant to infection during the first week of their use. Allografts are rejected and slough from the wound bed approximately 7-10 days after their placement due to immunologic incompatibility between the burn victim and the cadaver donor.

Despite their effectiveness, allografts also have certain disadvantages, including limited availability, cost considerations and the possibility of transmitting human diseases. Xenografting provides a potentially valuable alternative strategy for temporary coverage.

The purpose of this study was to evaluate the effectiveness of a novel approach to xenografting severely burned warfighters using genetically-modified porcine skin grafts. We examined whether these porcine skin grafts would perform as well as allogeneic skin grafts as a temporary biologic cover for severe burn injuries. These genetically-modified porcine skin grafts would provide significant advantages to the current gold standard with regard to availability, cost, safety and ethical considerations. This novel therapy represents a rapidly translatable product that could serve as immediately-available grafts for the initial treatment of severe battlefield burns and full-thickness skin loss from blast injury.

The concept of clinical utilization of xenografts is not new. Until now, however, porcine skin grafts have not been true, viable grafts. While they attach to the skin bed, they do not truly engraft, since no blood vessels connect the porcine skin to the underlying bed. In viable skin grafts, there is a rapid connection of blood vessels in the bed to existing vessels in the skin, followed only later by the slower invasion of the graft by additional host blood vessels This lack of true engraftment has limited the usefulness of living porcine grafts, since the lack of blood supply soon lead to desiccation and avascular necrosis. Failure of vascularization of xenografts is largely due to hyperacute

rejection, an immediate attack on the endothelium of graft blood vessels mediated by the recipient's preformed antibodies against antigens present on cells of the pig. The responsible antigen is the α-1,3-galactose (GalT) moiety found on the cell membrane of all species except humans and Old World primates. Preformed antibodies against this Gal epitope, which are present in large numbers in primates, immediately bind to this antigen. Upon binding, a cytotoxic cascade is initiated, aided by complement, causing immediate rejection by occluding graft vessels. Our genetically-engineered swine that we propose to use here, will avoid this complication.

We anticipate several major advantages to the use of porcine skin as temporary grafts for human burn victims: 1) porcine skin is considerably less expensive than human cadaver skin; 2) the source is readily available, since pigs are easily bred; 3) porcine skin is very similar to human skin histologically and functionally; 4) porcine skin carries far less risk of disease transmission than human skin, both because swine can be raised hygienically and because they share far fewer pathogens; and 5) pig skin possesses some of the same practical advantages as human skin in being simple to harvest and being easily stored, shipped, and later prepared quickly for use.

Until now, however, porcine skin grafts have not been true, viable grafts. While they attach to the skin bed by means of granulation tissue, they do not truly engraft, since no blood vessels connect the porcine skin to the underlying human skin bed. In viable skin grafts, there is a rapid inosculation (connection) of blood vessels in the bed to existing vessels in the skin, followed only later by the slower invasion of the graft by additional host blood vessels This lack of true engraftment has limited the usefulness of living porcine grafts, since the lack of blood supply soon lead to desiccation and avascular necrosis.

Failure of vascularization of xenografts is largely due to hyperacute rejection, an immediate attack on the endothelium of graft blood vessels mediated by the recipient's preformed antibodies against antigens present on cells of the pig. The responsible antigen is the α-1,3-Galactose (Gal) moiety found on the cell membrane of all species except humans and Old World primates. Preformed antibodies against this Gal epitope, which are present in large numbers in primates, immediately bind to this antigen. Upon binding, a cytotoxic cascade is initiated, aided by complement, causing immediate rejection by occluding graft vessels.

In order to avoid this complication, we have recently produced swine which do not express the Gal epitope (GalT-KO swine). Using a fibroblast line derived from one of our most highly inbred lines of miniature swine, the α-1,3-galactosyltransferase gene was disrupted through homologous recombination. The corresponding knockout animals (GalT-KO) were then produced by nuclear transfer. The availability of these animals now makes it possible to carry out pig-to-baboon xenografts in the absence of effects of natural anti-Gal antibodies. In the absence of the

hyperacute rejection engendered by these antibodies, hyperacute rejection should be eliminated. Moreover, although humans will still have antibodies against non-Gal antigens in pigs, studies in our laboratory with the transplantation of other organs have confirmed that these anti-non-Gal antibodies are no more prevalent or toxic than alloantibodies against other humans. Therefore, one might expect xenogeneic skin from swine to engraft on primates in a similar fashion to allogeneic skin. Engraftment would entail both a blood supply for the new skin as well as incorporation into the underlying skin bed (as opposed to the more flimsy attachment by granulation tissue currently seen). The net result of using GalT-KO skin would be a healthier, more normal graft that would last longer than Gal-positive pig skin grafts. It would also be less expensive and more readily available than human skin and other organic options. A herd of appropriate skin graft donor animals could be maintained for this purpose and could provide an attractive alternative to cadaveric skin as an emergency temporary graft in battlefield.

The purpose of this study was to compare the survival of skin grafts to baboons from GalT-KO swine to the survival of skin grafts from unmodified (i.e. GalT+ wildtype) swine or from allogeneic baboons and study the response of these grafts by gross examination, by histology and evaluation of the cellular and humoral immune responses evoked. Specifically we evaluated the effect of immunosuppression on outcome, the role of graft technique on outcome and the effect of skin treatment and storage on the effectiveness of the graft.

2. RESULTS

Clinical and Histopathologic Data
GalTKO Xenografts Exhibit Comparable Survival to Allogeneic Grafts (Confirmation of Preliminary Data and Reproducibility of Xenotransplantation Model): Our first set of experiments was designed to replicate preliminary, recently published data. Two baboons, B266 and B267, were transplanted. Split-thickness recipient wounds were prepared with a dermatome, and the fresh, split-thickness grafts from the 4 skin sources (**Self** baboon, **Allogeneic** baboon, **Gal+** swine and **GalT-KO** swine) were placed. B266 received no immunosuppression, while B267 received cyclosporine. The results confirmed our initial findings and clearly demonstrated the reproducibility of this xenotransplantation model. In both B266 (no immunosuppression) and B267 (CyA), the Gal+ skin was rejected within 4 days, appearing as a "white graft" that had not vascularized. By contrast, in baboon B266, the GalT-KO skin graft appeared to remain partially viable by postoperative day 7, similar to the allogeneic skin graft. Both were beginning to show signs, both by gross inspection and histopathologically, of rejection, and by day 11, both were completely rejected.

Results were similar for B267, with possibly a slightly extended time course for survival of both allogeneic

and GalT-KO skin grafts, likely due to the effect of the cyclosporine. By day 11, both were rejected, however, both clinically and histologically. Histology exhibited healthy dermis and epidermis on self skin and complete vacuolization of dermis- consistent with complete rejection- of Gal+ skin by POD 7. In contrast, early rejection of both GalT-KO and Allo grafts were observed at POD 7. Figure 1 exhibits the clinical results of a representative experiment in our model.

Multiple Grafts Do Not Affect Graft Survival: The next experiments were designed to examine whether rejection of a Gal+ skin graft, or of an allogeneic baboon skin graft, could influence the survival of a GalT-KO skin graft on the same animal. We evaluated clinical, histopathological and in vitro immunologic data on the next two animals to examine whether there was evidence to support this hypothesis. Note that, by not grafting Gal+ skin onto this baboon, we eliminated the possibility of a clinical immunologic effect on the GalT-KO graft secondary to increased inflammatory mediators that may occur from the rejection of a Gal+ graft in the same individual baboon. We found no such effect: the GalT-KO and Allo skin grafts behaved similarly to the previous two baboons, again both surviving intact until day 7.

Conversely, in animal B269, we tested the effect of Gal+ rejection on the baboon's immunologic response to the Allogeneic graft. Again, we found no such effect: the Allo skin graft survived intact until day 7. These results suggested that a more efficient, 4-grafts-per-animal approach might allow more rapid accumulation of data with more efficient use of animals. In addition, the self and Gal+ grafts would provide an advantage of an internal control in each animal both clinically and immunologically.

Confirmation of Second-Set Rejection, Processing of Frozen Grafts, Grafting on Split-Thickness Wound Beds: The next set of experiments was designed to assess whether humoral sensitization and rejection proceed identically for a second set of skin grafts of Allo and GalT-KO grafts given to a previously grafted baboon (i.e. *regrafting* baboons B266 and B267). In other words, what is the relative sensitization potency of Allo vs. non-Gal antigens once they have been "seen" by a primate's immune system? The answer to this question would have potential implications for clinical use of the GalT-KO skin grafts if a second graft was necessary on a burned soldier, and suggests another possible use for GalT-KO skin- a combination approach- when cadaver skin is also available to treat a soldier. For example, if a second graft was necessary for a burned soldier, after an initial cadaver graft had been placed, knowledge that the patient would be not have been sensitized to non-Gal antigens by the cadaver skin graft- a phenomenon that has been demonstrated for solid organ xenotransplantation- would allow use of GalT-KO skin *after* the cadaver graft sloughs (or vice versa), thereby buying time for the metabolic recovery of the

patient prior to definitive wound coverage. This treatment algorithm has significant potential for improved outcomes in severe burns.

In addition, we utilized these animals as an opportunity to acquire early data regarding two other variables, processing of frozen skin grafts, and grafting on a full-thickness wound bed, both of which are important military practicalities. We regrafted baboons B266 and B267. We observed rapid rejection of both Allo and GalT-KO second-set skin grafts, with no difference in pace of rejection. Grafts were rejected by day 4. We noted no difference between fresh and frozen grafts in terms of early take, and graft take was excellent on a full thickness wound bed (evidenced by self grafts).

Confirmation of Second-Set Rejection, Processing of Frozen Grafts, Grafting on Full-Thickness Wound Beds: To confirm that the processing of frozen grafts has no effect on GalT-KO skin viability, and that GalT-KO skin grafts will take on full-thickness wound beds as well as split-thickness, we performed autografts on GalT-KO pigs (i.e. pig-to-self-pig) on both full and split-thickness wound beds comparing fresh and frozen skin. We performed these experiments to assure that the rapid rejection of second-set grafts was due to sensitization and not to technical factors regarding our freezing protocol or wound bed preparation (theoretically, grafts should take equally well on split or full thickness wound beds). We observed no differences, as all grafts healed successfully.

Xenoskin Transplantation is Equally Effective on Full-Thickness Wound Beds: The next set of experiments was designed on *full-thickness* wound beds, representative of injuries requiring grafting in the field. Two baboons, B280 and B282, were transplanted. Full-thickness recipient wounds were created with a scalpel, and the different, fresh, split-thickness grafts from the 4 skin sources (**Self** baboon, **Allogeneic** baboon, **Gal+** swine and **GalT-KO** swine) were placed. B280 received no immunosuppression, while B282 received cyclosporine. The results of these experiments were consistent with our initial findings on split-thickness wound beds and clearly demonstrated the reproducibility of our xenotransplantation model in clinically-relevant *full-thickness* wounds. In both B280 (no immunosuppression) and B282 (CyA), the Gal+ skin was rejected within 4 days, appearing as a "white graft" that did not vascularize. By contrast, in baboon B280 the GalT-KO skin graft was viable at postoperative day 7, similar to the allogeneic skin graft. Both were beginning to show signs of rejection both visibly and histopathologically, and by day 11, both were rejected, as previously found. Results were similar for B282, in which both the GalT-KO and allogeneic skin were clinically viable at postoperative day 7.

Frozen Xenografts Are Effective on Full-Thickness Wound Beds: We next examined fresh vs. frozen grafts on full-thickness wound beds. Two baboons, B283 and B285,

were transplanted. Full-thickness recipient wounds were created with a scalpel, and the different, fresh or frozen split-thickness grafts (that had been previously harvested and frozen one week preoperatively) from the 4 skin sources (**Self** baboon, **Allogeneic** baboon, **Gal+** swine and **GalT-KO** swine) were placed. Neither B283 nor B285 received immunosuppression. The results again demonstrated that both fresh and frozen xenografts and allografts enjoyed comparable survival on full thickness defects. Results were similar for B283 and B285, in which both the fresh and frozen GalT-KO and allogeneic skin grafts were clinically viable at postoperative day 7. Control self grafts showed 100% acceptance and survival and Gal+ grafts again failed to engraft, appearing as "white grafts". In vitro analysis is also underway in these experiments.

These data have demonstrated that 1) GalT-KO skin xenotransplants from pig-to-baboon last at least as long as baboon allogeneic skin transplants; 2) second-set grafts reject rapidly, typical of a sensitized immune response; 3) graft survival is unaffected by freezing/thawing; and 4) graft survival is comparable on partial-thickness and full-thickness recipient wound beds.

Summary of Clinical and Histopathologic Data

a) Baboons B266, B267 (1st grafting):
- GalT-KO and Allo both remained intact until rejection between POD 7 and 11, per clinical and histologic findings
- Cyclosporine prolonged survival of both GalT-KO and Allo grafts
- Controls: Self (no rejection) and Gal+ skin (the Gal+ graft rejected in a hyperacute fashion, as predicted: the Gal+ graft was a "white graft" by POD4, suggesting that it never vascularized because the vasculature was destroyed due to naturally-present, pre-formed anti-Gal antibodies in the baboon that initiated the complement cascade and endothelial cell destruction)

b) Baboons B268, B269:
- GalT-KO and Allo rejected between POD 7 and 14
- Gal+ skin did not affect the survival of GalT-KO or Allo skin
- Controls: Self (no rejection) and Gal+ skin (POD 4 white graft again)

c) Baboons B266, B267 ("2nd set" grafting (Regrafting following sensitization by the first set of grafts)):
- GalT-KO and Allo rejected by POD 4 (more quickly than in the first set in which they rejected somewhere between POD 7 and 11
- Neither frozen/thawed grafts nor full thickness wound beds detrimentally effected early survival of the grafts
- Cyclosporine had no effect on the 2nd set rejection time of both GalT-KO and Allo grafts

d) Pig-to-pig Split-thickness wound beds, fresh vs. frozen GalT-KO autografts
-Freezing and thawing of grafts did not detrimentally affect survival of the autografts

e) Pig-to-pig Full-thickness wounds beds, fresh vs. frozen GalT-KO autografts
-Neither freezing and thawing nor use of full thickness wound beds detrimentally affected survival of grafts

f) Baboons B280, B282
-Full thickness wound beds did not detrimentally affect survival of the xenogeneic Galt-KO skin grafts

g) Baboons B283, B285
-Freezing and thawing of grafts did not detrimentally affect survival of the xenogeneic Galt-KO skin grafts

In Vitro Data
We have demonstrated that primate recipients of skin grafts from pigs that express the Gal antigen (i.e. Gal+ pigs) reject these grafts in a hyperacute manner, consistent with the presence of anti-Gal antibodies that cause hyperacute rejection of pig organ xenografts transplanted to primates (baboons). FACS and ELISA assays showed: 1) Pre-formed anti-**Gal** IgM, IgG antibodies were present in baboon, (which presumably explains hyperacute rejection of a Gal+ skin graft); 2) No pre-formed anti-non-Gal IgM, IgG antibodies were found (explaining the lack of hyperacute rejection of the GalT-KO skin graft); 3) No pre-formed anti-**Allo** IgM, IgG antibodies were present in baboon; and 4) No strong anti-allo Ab increase was observed following allo skin transplantation. See Figure 2.

Immunologic mechanisms involved in baboon responses to xeno vs. allo skin grafts were suggested by in vitro findings following second-set grafts. We demonstrated with baboons B266 (1st grafting: self, Gal+, GalT-KO, allo, no CyA)(2nd grafting: self, GalT-KO, allo, no CyA), B267 (1st grafting: self, Gal+, GalT-KO, allo, CyA) (2nd grafting: self, GalT-KO, allo, CyA):

a) T-cell responses (MLR)

i) 1st grafting - Allo response is stronger than Xeno *pre* skin transplant (Pre) (when MLR response is observed after a 2-day incubation. By day 5 the strong Allo response "exhausted" cells and displayed lower counts per minute). Xeno response was stronger than Allo response *post* skin grafting (POD 14, POD 21). CyA suppressed both Allo and Xeno responses (as shown by lower cpm (counts per minutes).

ii) 2nd grafting (post-sensitization) Xeno response stronger than Allo, and CyA had little effect (CyA dose may be too low to suppress a sensitized T-cell response).

b) B-cell responses (FACS, ELISA)

i) anti-Gal IgM, IgG (1st grafting): Anti-Gal IgM and IgG present pre-transplant (Day 0) , and IgG increased post grafting (Day 21) (suggesting that Gal antigens are strong enough to induce class switching pre-transplant, a finding that further demonstrates need for GalT-KO skin)

ii) anti-Gal IgM, IgG (2nd grafting): No change in anti-Gal IgM (Day 55, POD14, while IgG increased post second grafting (Day 55, POD14). For animal B268 no anti-Gal antibodies were found- as expected since only GalT-KO (and no Gal +) skin was grafted.

iii) anti-nonGal IgM, IgG (1st grafting)
- Lack of anti-nonGal IgM and IgG pre-transplant (suggesting that non-Gal antigens are not present pre-transplant.
-Anti-nonGal IgM to IgG switch post-skin transplant. IgG peak by POD7 (one week earlier than following the first set of skin grafts, suggesting sensitization to non-Gal antigens).

iv) anti-nonGal IgM, IgG (2nd grafting)
- anti-nonGal IgM, IgG present prior to 2nd grafting (~5 weeks post 1st grafting), increased post 2nd grafting to max POD 14 for IgM (likely secondary to sensitization from 1st grafting) and peaked at POD7 for IgG.

v) anti-allo IgM, IgG (1st grafting): No anti-allo IgM or IgG pre-transplant. Mild sensitization at a B cell level is observed following allogeneic skin transplant (suggesting an important T cell component in the rejection of allo skin)

vi) anti-allo IgM, IgG (2nd grafting): No anti-allo IgM or IgG present prior to second skin grafting (interesting that allo responses induced after 1st grafting are eventually lost, ~ 5 weeks after 1st grafting. Contrasts with anti-Gal and anti-nonGal which were still present at 5 weeks post 1st grafting).

vii) CyA effect on B-cell responses - CyA did not affect the B-cell responses appreciably (suggesting that either CyA dose was too low, or B-cell responses were not significantly affected by the T-cell responses to these skin grafts).

We have demonstrated with baboons B268 (self, GalT-KO allo, CyA), B269 (self, Gal+, allo, CyA):

a) T-cell responses (MLR):

Pre-transplant allo response was greater than xeno response (consistent with B266, B267 data); Post-transplant xeno response increased and was similar to allo response by POD 30.

i) Baboon B268(self, GalT-KO, allo, CyA):

- **anti-Gal IgM, IgG:** Present pre-transplant, minor increase post-transplant of IgM and higher increase of IgG. The increase of antibodies is probably due to the presence of many different antigens (minor antigens that are non-Gal) on the regular Gal + cells used for the assays).

- **anti-nonGal IgM, IgG:** present pre-transplant, increased post-transplant with max POD 14.

- **anti-allo IgM, IgG:** no antibody pre-transplant, increased post-transplant max POD 14-21.

ii) Baboon B269 (self, Gal+, allo, CyA):

- **anti-Gal IgM, IgG:** Present pre-transplant, and increases post transplant to max POD 14.
- **anti-nonGal IgM, IgG:** present pre-transplant, increases post-transplant with max POD 14.
- **anti-allo IgM, IgG:** no antibody pre-transplant, increases post-transplant max POD 14-21.

Summary of In vitro data:

1) Primary grafting of Gal+ skin to baboons led to hyperacute rejection of the grafts (white graft), due to high levels of natural anti-Gal antibodies. Levels of anti-Gal antibodies increased after rejection and second grafts were likewise hyperacutely rejected. Figure 2 a, b.

2) Primary grafting of GalT-KO skin to baboons led to rejection in approximately the same time frame as did grafting of allogeneic skin, and was followed by high B cell response, as indicated by early, specific Ab increase after rejection of the skin graft. The second graft underwent hyperacute rejection (white graft), presumably due to the high levels of anti-non-Gal antibodies produced after rejection of the first graft. Figure 2 c,d.

3) Primary grafting of allogeneic baboon skin to baboons led to rejection in approximately the same time frame as did grafting of GalT-KO skin, but was followed by a strong T cell response but a less vigorous B cell response, as indicated by a slower and smaller early, specific Ab increase after rejection of the skin graft. The second graft underwent accelerated but not hyperacute rejection. Figure 2e.

3. CONCLUSIONS

We have shown that primate recipients of skin grafts from pigs that express the Gal antigen (Gal+) reject these grafts in a hyperacute manner, consistent with the presence of anti-Gal antibodies responsible for hyperacute rejection of pig organs transplanted to primates (baboons). Thus, these unique, genetically engineered GalT-KO pigs will likely be required for success of pig skin xenografts.

We have demonstrated that primates do not exhibit this hyperacute rejection phenomenon when GalT-KO pig skin grafts are transplanted. In contrast, these xenotransplants last at least as long as primate skin allotransplants on split-thickness wounds. This result confirms our preliminary data and has important implications for the use of GalT-KO pig skin grafts to treat battlefield injuries.

We have demonstrated that GalT-KO swine skin can cover full-thickness wound beds (analogous to those expected in battlefield wounds) in primates equally as well as allogenieic skin grafts. Previous studies used split thickness wound beds. Data from full-thickness wound beds are comparable to split-thickness data. Histologically, the full-thickness bed is a better model, as analysis of these wounds can be performed without the confounding artifact of migration of peripheral skin cells into the wound area during healing. In addition, full-thickness wounds better represent the clinical situation, where 3rd and 4th degree burns require immediate treatment.

We have demonstrated no appreciable difference in graft function between fresh vs. frozen and thawed skin grafts from either swine or baboon sources. Thus frozen/thawed skin lasts at least as long as fresh skin.

We have elucidated possible differential immunologic mechanisms involvied in the response to xeno vs allo skin grafts following first or second transplants: Both Gal KO and Gal+ skin regrafting in sensitized animals led to high B cell responses, as indicated by early, specific Ab increases, resulting in hyperacute rejection (white graft). Allo skin regrafting in sensitized animals was followed by higher T cell responses and lower B cell responses than GalT-KO regrafting, as indicated by markedly increased T cell responsiveness without a correspondingly high, early Ab increase. The increased T cell responsiveness was likely responsible for accelerated rejection, but not a white graft.

These experiments suggest the following: 1) genetically-modified pigskin grafts should perform as well as human cadaveric allogeneic skin grafts as a temporary biologic cover for severe burn injuries; 2) GalT-KO skin grafts function well after freezing and thawing; 3) GalT-KO skin grafts provide an effective cover for the full-thickness skin injuries that are typically seen in burn injuries sustained in combat; 4) a short course of immunosuppression may enhance the duration and quality of these grafts; and 5) in vitro findings suggesting that antibody responses are likely to be more prevalent in the rejection of GalT-KO skin than in the rejection of allogeneic skin – suggesting that targeting of the B cell response to non-Gal antigens may improve results of GalT-KO skin grafts.

Our data demonstrate the utility of GalT-KO xenogeneic skin grafts under a number of different experimental circumstances. Based on these results, we envision a frozen, readily-available military burn dressing, capable of being transported in a medic's pack, that could be used as a lifesaving temporary skin graft for immediate, sterile coverage of critically-injured areas of a blast-wounded or flame-burned soldier's body. The ability to quickly cover such wounds would prevent life-threatening infection and fluid/electrolyte loss while the combatant is evacuated to tertiary-care centers for definitive treatment. The Gal-KO pig skin graft would provide wound coverage for as many as 7-10 days post-injury before requiring definitive replacement with a permanent autograft. The treatment approach would replace or provide an adjunct to the current method of utilizing human cadaver allograft skin as a temporary dressing- an extremely effective technique that is underutilized due to a lack of availability, portability, cost-effectiveness, as well as ethical and infectious disease concerns associated with the use of human tissue.

In addition, previous studies of responses to allogeneic vs. xenogeneic transplants make it likely that neither GalT-KO nor allogeneic skin grafts will sensitize for each other, so that sequential grafts may be possible, thereby extending total survival of the temporary cover for over two weeks. A herd of appropriate skin graft donor animals could be maintained for this purpose and could provide an attractive alternative to human cadaveric allograft skin as an emergency temporary graft. Since human cadaveric allogeneic skin grafts are the current "gold standard" for temporary skin grafts and since our genetically-modified pig skin would have significant advantages related to availability, cost, and safety, we intend to develop this model further as a new approach to the initial treatment of severe battlefield injuries.

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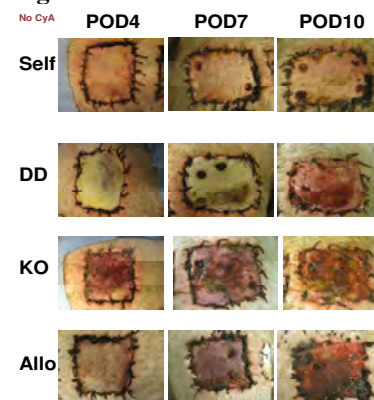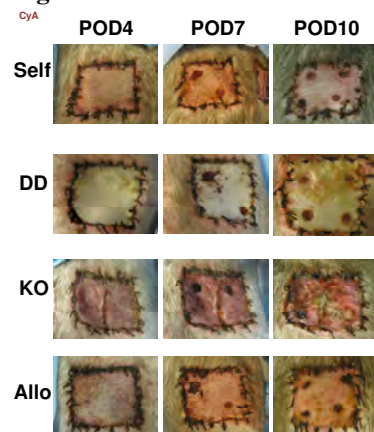
Figure 1a

Figure 1b
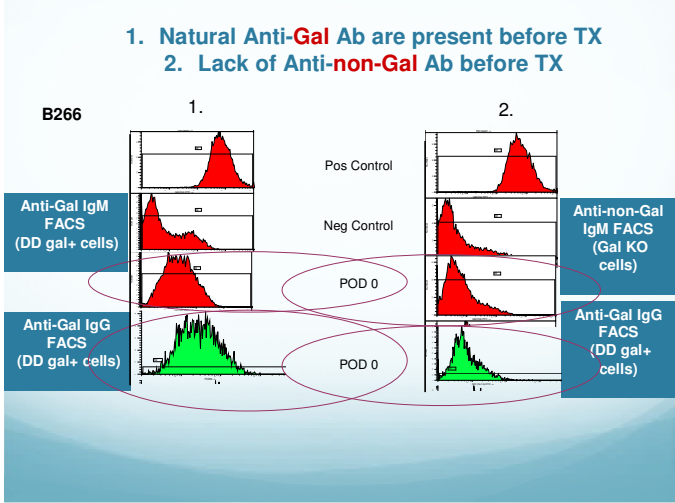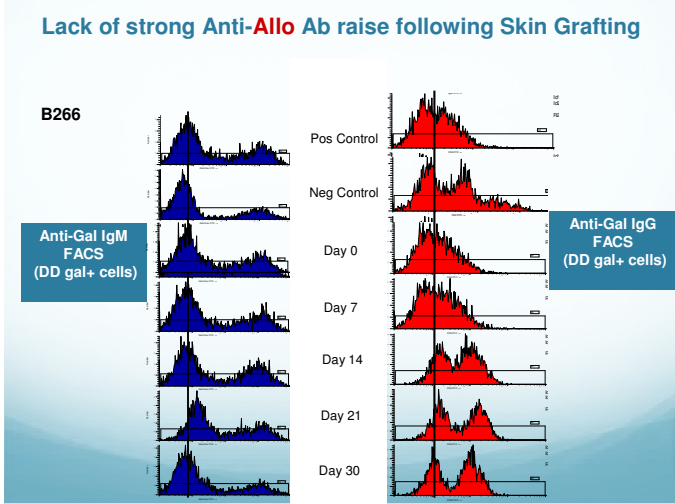
Figure 2a

1. **Natural Anti-Gal Ab are present before TX**
2. **Lack of Anti-non-Gal Ab before TX**

Figure 2b

Lack of strong Anti-Allo Ab raise following Skin Grafting

Figure 2c

Figure 2d

Figure 2e